Gamma-BUTYROBETAINE HYDROXYLASE ORIGINATED FROM NEUROSPORA CRASSA

Technical Field

The present invention relates to γ-butyrobetaine hydroxylase (γ-BBH) originating from *Neurospora crassa*. More particularly, the present invention relates to a polynucleotide encoding γ-butyrobetaine hydroxylase originating from *Neurospora crassa*, a recombinant vector comprising the polynucleotide, a transformant transformed with the recombinant vector, γ-butyrobetaine hydroxylase encoded by the polynucleotide, and a method of preparing L-carnitine by hydroxylating γ-butyrobetaine using γ-butyrobetaine hydroxylase encoded by the polynucleotide.

Background Art

L-carnitine (3-hydroxy-4-trimethylamino-butyrate), which is also known as vitamin BT, is a natural vitamin analog that is very important in human metabolism. L-carnitine was originally isolated from bovine muscle tissue in 1905 by two Russian scientists, Gulewitsch and Krimberg, and its chemical structure was identified in 1932. L-carnitine is found in nearly all cells of the body and transports activated free long-chain fatty acids across the

inner membrane of the mitochondria. Since the inner mitochondrial membrane is an impenetrable barrier to acyl-CoA esters, free long-chain fatty acids, activated to acyl-CoA esters in the cytoplasm, pass across the membrane when esterified to L-carnitine. When L-carnitine is present in low levels in the skeletal muscles, liver, heart and kidneys, free long-chain fatty acids are difficult to utilize as an energy source. This abnormal carnitine metabolism causes various diseases, including growth retardation, cardiomyopathy and muscle weakness. When L-carnitine is not synthesized in suitable amounts in the body, carnitine should be absorbed from foods to avoid carnitine deficiency symptoms. Especially in infants who are not able to biosynthesize L-carnitine, L-carnitine is an essential nutrient.

L-carnitine is used as an active component in pharmaceutical preparations. Exogenous supplementation of L-carnitine is required to treat carnitine deficiency and other diseases, especially cardiac diseases. Recently, this therapeutic use of L-carnitine has become increasingly important (R.A. Frenkel and J.D. Mc Garry, "Carnitine biosyntheis, metabolism and functions", Academic Press, 1980).

L-carnitine has been identified as playing many important roles in the body. However, conventional methods including biological extraction are not suitable for mass

production of L-carnitine. One method capable of easily obtaining L-carnitine is to utilize DL-carnitine including optical isomers. This method causes side effects in the body because it contains D-carnitine (Curr. Ther. Res. 28, 195-198, 1980). In many cases, D-carnitine competes with L-carnitine in the body and interrupts the mitochondrial beta-oxidation of free long-chain fatty acids. In patients having remarkably reduced renal function, this impaired metabolism of long-chain fatty acids leads to more serious inhibition.

Many efforts have been made to obtain optically pure L-carnitine, which include a chemical optical resolution method (US Patent No. 5,166,426), a biological method using microorganisms or enzymes (US Patent No. 5,187,093), and a method of producing L-carnitine using a chiral compound as a starting compound (US Patent No. 6,420,599 B2).

Among various methods for obtaining L-carnitine, a biological method using microorganisms or enzymes employs a biological enzyme, gamma-butyrobetaine hydroxylase, to produce optically active L-carnitine. This enzyme was isolated in mice and humans (Rebouche and Engel, *J Biol Chem* 255:8700-8705, 1980), and its nucleotide sequence was identified. Higher organisms including mammals utilize an amino acid residue of proteins, lysine, as a precursor for L-carnitine biosynthesis, whereas *Neurospora crassa* produces optically pure L-carnitine from free lysine (Fraenkel, *Biol Bull*, 104:359-371, 1953). The mechanism of L-carnitine

biosynthesis is briefly as follows. Carnitine synthesis begins with methylation of lysine by S-adenosylmethionine acting as a methyl donor, resulting in the formation of ε-N-trimethyllysine. Trimethyllysine is enzymatically transformed into β-hydroxy-trimethyllysine. From the synthesized β-hydroxy-trimethyllysine, trimethylaminobutyl aldehyde is formed, and is then converted to γ-butyrobetaine.

A nucleotide sequence encoding γ-butyrobetaine hydroxylase, which is derived from *Neurospora crassa* and produces L-carnitine using γ-butyrobetaine, produced through the aforementioned mechanism, as a precursor, has not been identified prior to the present invention.

Disclosure of the Invention

Based on this background, the present inventors identified a new gene encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa*, and successfully produced L-carnitine from γ-butyrobetaine by a biological method employing γ-butyrobetaine hydroxylase expressed using the gene.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly

understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 shows the results of SDS-PAGE (polyacrylamide gel electrophoresis) of centrifugal supernatants of cell lysates of *Escherichia coli* (*E. coli*) BL21 not containing the γ-butyrobetaine hydroxylase (γ-BBH) gene and *E. coli* BL21 containing the γ-BBH gene and induced by IPTG (Isopropyl-β-D-thiogalactopyranoside);

FIG. 2 shows the results of 0.8% agarose gel electrophoresis of an amplified γ-BBH cDNA gene cloned into pT7-7;

FIG. 3 is a multiple sequence alignment in which an amino acid sequence of γ-BBH from *Neurospora crassa* is aligned against that of human, rat and *Pseudomonas*-derived γ-BBH;

FIG. 4 is a schematic presentation of a pT7-BBH2 plasmid; and

FIG. 5 is a schematic diagram of a process of producing L-carnitine from γ-butyrobetaine.

Best Mode for Carrying Out the Invention

In one aspect, the present invention provides a gene encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa*, the gene represented by SEQ ID NO. 1.

In order to obtain a gene encoding γ-butyrobetaine

hydroxylase derived from the filamentous fungus *Neurospora crassa*, the present inventors first compared heterogeneous genes encoding γ-butyrobetaine hydroxylase to find conserved regions. A homology search was conducted between the conserved regions and the entire gene sequences of *N. crassa*, registered in the gene database. From genes having partially similar sequences, a candidate gene displaying γ-butyrobetaine hydroxylase activity was selected. In order to clone the candidate gene, primers specific for the gene were synthesized. A *Neurospora crassa* cDNA library was prepared and screened for the target gene using the synthesized primers. The thus obtained cDNA clone was inserted into a suitable vector. The resulting recombinant vector was transformed into *Escherichia coli*, and gene cloning was found to be successful by experiments using the transformant. Protein expression of the gene carried by the recombinant vector was induced by IPTG treatment and analyzed by SDS-PAGE. Compared to a control, an *E. coli* transformant displaying specific protein expression was found to produce L-carnitine using γ-butyrobetaine as a substrate (Table 1).

The present invention is based on the finding of a novel gene encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa*, the gene being identified as described above and being not identified prior to the present invention. A polynucleotide sequence of γ-butyrobetaine

hydroxylase derived from *Neurospora crassa*, which has been newly identified by the present inventors, is represented by SEQ ID NO. 1.

Variants, for example, fragments and derivatives, of the polynucleotide of SEQ ID NO. 1 encoding a polypeptide having γ-butyrobetaine hydroxylase activity are also included within the scope of the present invention as long as they are expressed in a form containing a gene having the polynucleotide sequence of SEQ ID NO. 1.

In another aspect, the present invention provides a polynucleotide encoding a protein that has 70% or higher homology to the polynucleotide of SEQ ID NO. 1 and has γ-butyrobetaine hydroxylase activity.

The term "homology", as used herein for a polynucleotide sequence or a protein or polypeptide encoded by the polynucleotide sequence, indicates sequence similarity between wild-type amino acid sequences or wild-type nucleotide sequences. In the case of a protein, "homologous" includes an amino acid sequence 75% or higher, preferably 85% or higher, more preferably 90% or higher and even more preferably 95% or higher identical to the amino acid sequence of a γ-butyrobetaine hydroxylase protein according to the present invention. Typically, a protein homologue may include an active site identical to a target protein. In the case of a gene, "homologous" includes a gene sequence 75% or higher, preferably 85% or higher, more

preferably 90% or higher and even more preferably 95% or higher identical to a polynucleotide sequence encoding a γ-butyrobetaine hydroxylase protein according to the present invention. The homology evaluation may be done with the naked eye or using a commercially available program. Using a commercially available computer program, the homology between two or more sequences may be expressed as a percentage (%), and the homology (%) between adjacent sequences may be evaluated.

In a preferred aspect, the present invention provides a polynucleotide encoding a protein that has a homology of 75% or higher, preferably 85% or higher, more preferably 90% or higher and even more preferably 95% to the sequence of SEQ ID NO. 1 and has γ-butyrobetaine hydroxylase activity.

In a further aspect, the present invention provides a polynucleotide encoding γ-butyrobetaine hydroxylase represented by SEQ ID NO. 2.

γ-butyrobetaine hydroxylase may be produced in a large scale according to the present invention by inserting a polynucleotide gene encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa* into a vector and inducing expression of the protein using the resulting recombinant vector.

Thus, in yet another aspect, the present invention provides a recombinant vector comprising a polynucleotide

gene encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa*.

The term "vector", as used herein, refers to a DNA construct that contains a DNA sequence operably linked to regulatory sequences capable of controlling the expression of a protein in a suitable host and sequences introduced for facilitating other genetic manipulation or optimizing the expression of the protein. Such regulatory sequences include a promoter for transcription control, an operator selectively added for transcription control, a suitable mRNA ribosome binding site and sequences controlling termination of transcription/translation. Such a vector for insertion of an exogenous gene may be a plasmid, a virus, a cosmid, or the like.

The vector includes cloning vectors and expression vectors. The cloning vector is a replicable plasmid into which exogenous DNA is inserted, and delivers exogenous DNA into host cells transformed therewith. "Expression vector" typically means a carrier into which a fragment of exogenous DNA, generally a fragment of double-stranded DNA, is inserted. "Exogenous DNA" refers to heterogeneous DNA that does not naturally occur in host cells. The expression vector is able to replicate independently of host chromosomal DNA in host cells so that inserted exogenous DNA may be produced. As generally known in the art, in order to increase the expression level of a transfected

gene in a host cell, the gene should be operably linked to transcription and translation regulatory sequences functional in a host cell selected as an expression system.

A pT7-BBH2 vector (*Eshcherichia coli* DH5α CJ2004), which is constructed according to the present invention for expression of a polynucleotide encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa*, was deposited at an international depository authority, the Korean Culture Center of Microorganisms (KCCM) on January 27, 2004, and assigned accession number KCCM-10557.

In still another aspect, the present invention provides a transformant transformed with a recombinant vector comprising the gene.

The term "transformation", as used herein, means the introduction of DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration. Host cells useful for the transformation according to the present invention may be prokaryotic or eukaryotic. In addition, host cells having high introduction efficiency of foreign DNA and having high expression levels of introduced DNA may be typically used. Examples of host cells include prokaryotic and eukaryotic cells, such as bacteria, for example, *Escherichia* sp., *Pseudomonas* sp., *Bacillus* sp. and *Streptomyces* sp., fungi and yeast, insect cells such as *Spodoptera frugiperda* (Sf9), and

animal cells such as CHO, COS 1, COS 7, BSC 1, BSC 40 and BMT 10. *Escherichia coli* may be preferably used.

An amino acid sequence encoded by the polynucleotide of SEQ ID NO. 1 is represented by SEQ ID NO. 2. Thus, in still another aspect, the present invention provides γ-butyrobetaine hydroxylase derived from *Neurospora crassa* and having the amino acid sequence of SEQ ID NO. 2.

In still another aspect, the present invention provides γ-butyrobetaine hydroxylase selected from the group consisting of variants that have a homology of 75% or higher, preferably 85% or higher, more preferably 90% or higher and even more preferably 95% to the sequence of SEQ ID NO. 2 and have γ-butyrobetaine hydroxylase activity.

As shown in FIG. 5, the γ-butyrobetaine hydroxylase according to the present invention may be used to produce L-carnitine from γ-butyrobetaine, thereby obtaining optically pure L-carnitine.

Thus, in still another aspect, the present invention provide a method of preparing L-carnitine which comprises hydroxylating γ-butyrobetaine using the aforementioned γ-butyrobetaine hydroxylase.

The L-carnitine obtained as described above may be used for L-carnitine supplementation for treating carnitine deficiency and other therapeutic purpose.

A better understanding of the present invention may be obtained through the following examples which are set

forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Construction of *Neurospora crassa* cDNA library

In order to obtain cDNA of *Neurospora crassa*, mRNA was first isolated from *Neurospora crassa*, and cDNA was synthesized from the isolated mRNA by PCR (Polymerase Chain Reaction) using a polyT primer. cDNA was inserted into EcoRI/XhoI sites of an AD5 cloning vector, and a cDNA pool constructed in a plasmid was prepared as follows. An *Escherichia coli* strain BNN322 was cultured in LB medium supplemented with kanamycin and 0.2% maltose overnight, harvested by centrifugation, and suspended in 1 ml of 10 mM $MgSO_4$. The bacterial suspension was cultured with 3.5×10^7 λ phages possessing a cDNA pool for 30 min at 30°C without agitation. After 2 ml of LB medium was added to the culture, the infected strain was cultured for 60 min at 30°C with agitation. The resulting culture was smeared onto LB plates containing ampicillin (75 μl/ml). Plasmids were isolated from emerged colonies, thus creating a cDNA library pool.

EXAMPLE 2: Preparation of primers for obtaining γ-butyrobetaine hydroxylase gene

The amino acid sequence of *Neurospora crassa*-derived γ-butyrobetaine hydroxylase (γ-BBH) was compared with that of human, rat and *Pseudomonas*-derived γ-BBH (FIG. 3). Sequence 1 represents the amino acid sequence of *N. crassa*-derived γ-BBH, Sequence 2 for that of human-derived γ-BBH, Sequence 3 for that of rat-derived γ-BBH, and Sequence 4 for that of *Pseudomonas*-derived γ-BBH. Sequence homology results are as follows (Start of Pairwise alignments):

Sequences (1:2) Aligned. Score: 11%
Sequences (1:3) Aligned. Score: 11%
Sequences (1:4) Aligned. Score: 10%
Sequences (2:3) Aligned. Score: 88%
Sequences (2:4) Aligned. Score: 29%
Sequences (3:4) Aligned. Score: 29%.

N. crassa-derived γ-BBH was found to have a 11% homology to human-derived γ-BBH.

A set of primers, below, was designed for cloning *N. crassa*-derived γ-BBH based on sequence information of *N. crassa* genome.

Primer 1 (SEQ ID NO. 3):
5'- ATG AAT TCC ATA TGA TGG CCA CGG CAG CGG TTC AG -3'
Primer 2 (SEQ ID NO. 4):
5'- ATT AGT CGA CTC AAT ACC CTC CCC CAC CCT G -3'

EXAMPLE 3: Obtainment of γ-BBH-encoding gene

The γ-BBH gene was amplified from the *Neurospora crassa* cDNA library, prepared in Example 1, by PCR using a set of primers prepared in Example 2. The PCR product was electrophoresed on an agarose gel, and a band was observed at about 1.4 kb. The nucleotide sequence of the amplified gene was determined by automatic DNA sequencing. Also, the determined nucleotide sequence was subjected to homology searches for nucleotide sequences using the BLAST program from NCBI. As a result, a gene 100% identical to the amplified gene was found in the genome sequence of *Neurospora crassa*, and the found gene was mentioned for function of its translational product only as a hypothetical protein. Then, the PCR product was digested with both EcoRI and SalI, ligated with pUC19 digested with the same restriction enzymes, and introduced into *Escherichia coli* DH5. A transformant was identified by blue/white screening. When the plasmid was isolated from the transformant and analyzed, the γ-butyrobetaine hydroxylase gene was found to have been successfully inserted into the plasmid.

EXAMPLE 4: Construction of pT7-BBH2 plasmid

The obtained plasmid containing the γ-butyrobetaine

hydroxylase gene was digested with NdeI and SalI, electrophoresed on a low-melting agarose gel. The DNA fragment corresponding to the γ-butyrobetaine hydroxylase gene was excised from the gel, purified, and inserted into pT7-7 treated with NdeI and SalI (FIG. 4). The resulting plasmid was transformed into *Escherichia coli* DH5 and grown on solid plates containing ampicillin. From emerged colonies, the recombinant plasmid was isolated. When the recombinant plasmid was digested with NdeI and SalI, the γ-BBH gene was found to have been successfully inserted into the plasmid (FIG. 2). Thus, the recombinant plasmid was designated as "pT7-BBH2". This recombinant plasmid was introduced into *Escherichia coli* DH5α. The resulting transformant was designated as "*Escherichia coli* DH5α CJ2004", which was deposited at the Korean Culture Center of Microorganisms (KCCM) on January 27, 2004 and assigned accession number KCCM-10557 .

EXAMPLE 5: Transformation of the pT7-BBH2 plasmid into expression bacterial strain *Escherichia coli* BL21(DE3)

The pT7-BBH2 plasmid possessing an ampicillin selection marker was transformed into an expression bacterial strain, *Escherichia coli* BL21(DE3). The *E. coli* BL21(DE3) strain produces T7 RNA polymerase in the presence of lactose or IPTG, which induces the translation of the γ-

butyrobetaine hydroxylase gene. The transformed cells were smeared onto solid media containing ampicillin. From emerged colonies, the plasmid was purified and digested with NdeI and SalI to examine the size of the inserted gene and the plasmid. As a result, the pT7-BBH2 plasmid was found to have been successfully introduced into the E. coli strain BL21(DE3).

EXAMPLE 6: Expression of γ-butyrobetaine hydroxylase

The transformant BL21(DE3)/pT7-BBH2, which was prepared in Example 5 by transforming the pT7-BBH2 plasmid into *E. coli* BL21(DE3), was cultured to evaluate the expression of γ-butyrobetaine hydroxylase. The transformant was cultured in a 250-ml baffle flask containing 50 ml of LB medium or LB medium supplemented with ampicillin. When the culture reached an OD600 value of 0.6, 1 mM IPTG was added to the medium, and the cells were further cultured for 4 hrs. The cells were harvested by centrifugation at 4,000×g for 15 min and resuspended in 1 ml of lysis buffer (140 mM NaCl, 200 g/liter glycerol, 1 mM DTT, 10 mM sodium phosphate buffer, pH 7.4,). The cell suspension was placed in ice and ultrasonicated for 10 sec five times using an ultrasonicator to disrupt cells. Then, the disrupted cells were centrifuged at 10,000×g for 20 to 30 min at 4°C. The supernatant was recovered, and the cell debris was

discarded. SDS-PAGE analysis showed a band at about 49 kDa corresponding to the size of γ-butyrobetaine hydroxylase (FIG. 1). Protein concentrations were determined by the Bradford assay according to the intended use.

EXAMPLE 7: Measurement of L-carnitine

A crude extract of *Neurospora crassa* was incubated in 500 μl of assay buffer (20 mM KCl, 3 mM ketoglutarate, 10 mM sodium ascorbate, 2 g/liter Triton X-100, 0.25 mM $(NH_4)_2Fe(SO_4)_2$, 0.2 mM butyrobetaine, 20 mM potassium phosphate buffer, pH7.0) for 1 hr at 37°C. 500 μl of the supernatant of the extract was mixed with 500 μl of 1.2 M perchloric acid. The mixture was incubated for 10 min at room temperature and centrifuged for 5 min. 600 μl of the supernatant was mixed with 320 μl of 0.7 M K_3PO_4 and incubated in an ice bath for 20 min. After the mixture was centrifuged for 5 min, 750 μl of the supernatant was diluted in 250 μl of sterile distilled water. The diluted supernatant was supplemented with 100 μl of DNTB/H_2O_2 and incubated for 10 min at room temperature. Then, the reaction mixture was supplemented with 50 μl of a catalase solution, incubated at room temperature for 30 min, and centrifuged. 1 ml of the supernatant was mixed with 50 μl of acetyl CoA and incubated at room temperature for 10 min. Absorbance was measured at 405 nm and L-carnitine

concentration was computed.

EXAMPLE 8: Evaluation of *N. crassa*-derived γ-butyrobetaine hydroxylase for the ability to produce L-carnitine using γ-butyrobetaine as a substrate

The E. coli strain BL21(DE3), transformed with the γ-butyrobetaine hydroxylase gene in Example 5, was cultured in a 250-ml baffle flask containing 50 ml of LB medium or LB medium supplemented with ampicillin. When the culture reached an OD600 value of 0.6, 1 mM IPTG was added to the medium, and the cells were cultured for over 8 hrs at 25°C in order to prevent the formation of inclusion bodies while inducing the formation of accurate protein tertiary structure. Then, the cells were harvested by centrifugation at 4,000×g for 15 min, and a protein crude extract was prepared according to the same method as in Example 6. The crude extract containing 1.0 mg/ml proteins was incubated in a reaction buffer containing 0.5 mg/ml γ-butyrobetaine for 4 hrs. L-carnitine concentration was determined according to the same method as in Example 7, and the results are given in Table 1, below.

TABLE 1

Assay mixture	L-carnitine Conc. (μg/ml)
γ-BBH assay buffer + 1.0 mg/ml BL21(DE3) crude extract	0.0

γ-BBH assay buffer + 1.0 mg/ml BL21(DE3)/pT7-BBH2 (induced by IPTG) crude extract	0.8

Industrial Applicability

As described hereinbefore, the novel gene encoding γ-butyrobetaine hydroxylase derived from *Neurospora crassa* is useful for producing optically pure L-carnitine from γ-butyrobetaine.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. CJ Corp.
500 5-GA NAMDAEMUN-RO
CHUNG-KU, SEOUL
REPUBLIC OF KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : *Escherichia coli* DH5α CJ2004	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10557
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authouity accepts the microorganism identified under I above, which was received by it on Jan. 27. 2004. (date of the original deposit)[1]	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: Jan. 29. 2004

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authouity.

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